LIF: lots of interesting functions

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Leukaemia inhibitory factor (LIF) is one of a growing number of cytokines that cannot be readily categorized according to its functions. Rather, these pleiotropic hormones have diverse and often overlapping effects on a multitude of cell types: for example, LIF can inhibit the differentiation of embryonal stem cells on one hand and induce the differentiation of M1 leukaemic cells on the other. Recent work has shed light on the physiological effects of LIF, how these are limited, and the biochemical and biological properties of LIF and its receptor.

COMMUNICATION between cells is central to a wide variety of complex biological processes, such as embryonic development and defence against infection. One of the primary means by which cells communicate is via the secretion of polypeptide hormones that exert their actions through specific receptors expressed on the surface of responsive cells. The number of polypeptide hormones that have been biochemically isolated and/or genetically cloned has increased dramatically over the past decade. Rather than exerting a single biological effect, many hormones are pleiotropic in their action. Moreover, there is considerable redundancy of action between hormones with several eliciting apparently identical effects. The properties of pleiotropy and redundancy are extreme in the case of a recently described cytokine, leukaemia inhibitory factor (LIF; Fig. 1). LIF exerts a broad range of effects on many cell types, yet all but one of these actions is shared with other cytokines. This review focuses on the structure of LIF, its biological actions *in vitro* and *in vivo* and the properties of the LIF receptor. In addition, a model of receptor structure that suggests a mechanism for the pleiotropic and redundant nature of cytokines is discussed.

The LIF gene and protein

LIF has been purified and cloned independently by a number of groups on the basis of its action in many biological assays, ranging from the induction of monocytic differentiation in the leukaemic cell line M1, to the suppression of differentiation of totipotent embryonal stem (ES) cells. As a consequence, LIF is known by a plethora of synonyms (Table I; Ref. 1 and citations therein).

LIF mRNA is transcribed from a single copy gene, composed of three exons, which is located on similar regions of the mouse and human genome[2–6]. The first exon encodes the 5'-untranslated region of the message and the initial residues of the leader sequence. The remainder of the leader sequence and the first third of the mature LIF protein are encoded by the second exon, while the carboxy-terminal two-thirds of LIF and an extensive 3'-untranslated region are encoded by exon 3 (Ref. 6).

There are alternative copies of the first exon in the mouse. These are transcribed from separate upstream promoter regions[7], raising the possibility that differential spatial and temporal regulation of the expression of the two forms of LIF may occur. The resultant proteins differ only in the amino-terminal portion of the leader sequence. This difference has been claimed to specify the extracellular fate of the mature protein, with one form remaining free in solution and the other being associated with the extracellular matrix[7]. Since the leader sequence of both forms is presumably cleaved co-translationally, the mechanism by which they direct the fate of otherwise identical proteins remains unclear. The critical test of the presumptive extracellular sorting signals will be whether they specify the location of heterologous proteins, as is the case for nuclear and endoplasmic sorting signals[8].

The mature LIF protein is heavily glycosylated, almost exclusively on asparagine residues. Differential glycosylation accounts for both the variability in the relative molecular mass and charge of LIF (M_r 32 000–67 000 and *pI* 8.6 to >9.2). Interestingly, the *in vitro* function of LIF does not appear to be affected by the extent of glycosylation, with non-glycosylated recombinant LIF produced in *E. coli* (M_r 20 000) and hyperglycosylated recombinant LIF produced in yeast (M_r 250 000 to >450 000) exhibiting a similar specific activity[9]. Whether the glycosylation pattern of LIF influences its stability or function *in vivo* has yet to be determined.

Recently oncostatin-M, a cytokine which, like LIF, can induce the monocytic differentiation of the leukaemic cell line M1, has been shown to share a 30% amino acid sequence similarity with LIF[10]. In addition, the position and pairing of the four cysteinyl residues of oncostatin-M are similar to four of the six cysteinyl residues of LIF. The use of a pattern search algorithm, rather than a linear alignment algorithm, suggested that there are primary sequence and secondary structural similarities between LIF, oncostatin-M and ciliary neurotrophic factor (CNTF). LIF also appears related, though to a lesser degree to interleukin 6 (IL-6) and granulocyte colony-stimulating factor (G-CSF)[11].

Biological variety is the spice of LIF

The biological properties of LIF have been defined in terms of its actions *in vitro* and the pathological effects observed upon elevation of its circulating concentration in mice.

Technologically, and perhaps biologically, the most interesting effect of LIF is to be found upon early embryonic cells. Mammalian blastocysts are composed of two distinct cell types – the outer trophectoderm, which contributes to the formation of the placenta and an inner group of cells, known collectively as the inner cell mass (ICM). Cells of the ICM are totipotent, giving rise to the extra-embryonic membranes as well as to the foetus proper[12]. When cultured *in vitro*, in the absence of an exogenous stimulus, these cells lose their totipotency and differentiate into a wide variety of cell types. If, however, cells of the ICM are cultured on appropriate feeder layers[13], in the medium conditioned by such feeders[13] or, as has

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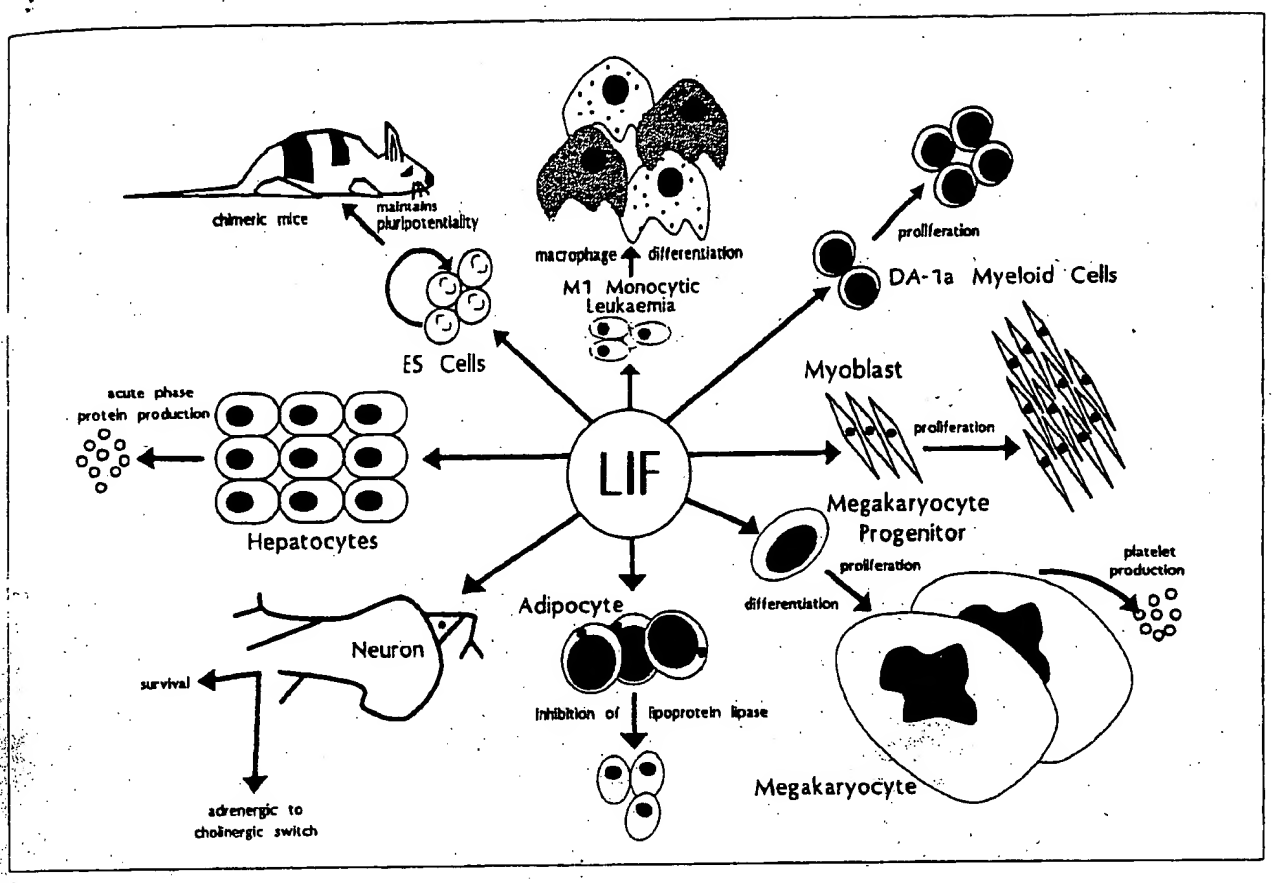

Figure 1

Pleiotropic nature of LIF's biological effects: The actions of LIF *in vitro* are extremely diverse and range from the induction of monocytic differentiation to the suppression of ES cell differentiation[1]. The parallel between LIF's actions *in vitro* and *in vivo* and its possible physiological roles are discussed in the text.

been shown recently, in the presence of purified LIF[9,15] they retain their pluripotentiality indefinitely and are known as embryonal stem (ES) cells. LIF is able to completely replace feeder layers, not only in the maintenance of previously established ES cell lines, but also in the isolation of new karyotypically normal cell lines that are capable of contributing with high efficiency to the formation of germ cells in chimeric mice[9,15,16].

The ability to maintain indefinitely the pluripotentiality of ES cells *in vitro* allows these cells to be manipulated genetically, for example using homologous recombination to mutate a specific gene. Genetically altered ES cells may then be used to generate mice that are chimeric both in somatic and germ tissue[13,14]. Since such mice will have germ cells that carry the mutation of interest, they may be mated to yield offspring that are heterozygous for the mutation and in turn these can be bred to give homozygotes. Using such technology the phenotype of null mutations of a wide range of genes, including those encoding homeobox, cytokine and tumour suppressor proteins, have been analysed (for example see Ref. 17).

ES cell culture systems have been developed in which, upon the withdrawal of LIF, efficient differentiation into haemopoietic tissue occurs in an environment similar to the visceral yolk sac[18]. Haemopoietic stem cells, present within this population, are capable of differentiation into mature blood cells of several lineages *in vitro* and reconstituting the haemopoietic system of lethally irradiated recipients *in vivo* (Ref 18; G. R. Johnson, pers. commun.). Clearly, if one produces haemopoietic stem cells from ES cells that have been genetically manipulated, and uses these to rescue lethally irradiated mice whose own stem cells have been destroyed, the direct effect of the mutation on haemopoiesis may be studied in isolation of any deleterious effects the mutation may have during embryonic development.

In contrast to its ability to inhibit the differentiation of ES cells, LIF induces the monocytic differentiation and suppresses the clonogenicity of the murine leukaemic cell line M1 (Ref. 1). In this regard it is functionally similar to the cytokines IL-6, G-CSF and oncostatin-M[19]. These apparently opposite effects of LIF occur at a similar concentration[9,19]. The contradictory nature of LIF's actions is also seen in its stimulation of the proliferation of several haemopoietic cell lines; this is in contrast to its suppression of M1 cell clonogenicity[1]. *In vitro*, LIF also enhances the mitogenic effect of interleukin 3 (IL-3) on megakaryocyte progenitors[20] and haemopoietic stem cells[21] and, even at low doses, is observed to increase the number of megakaryocyte progenitors, megakaryocytes and platelets *in vivo*, in mice and monkeys (Ref. 22; P. Mayer, pers. commun.). The proliferation of a number of other cell types including myoblasts is also enhanced by LIF[1]. A dramatic effect of LIF *in vivo* is a marked osteoblastosis and increase in bone deposition in the long bones of mice with elevated circulating LIF concentrations[22,23].

LIF also influences the function of a variety of terminally differentiated cell types such as neurons, hepatocytes

Table I. Defining properties and synonyms of LIF

Synonym[a]	Species	Defining biological activity	$M_r \times 10^{-3}$	Amino-terminal sequence
LIF	Murine	Induction of M1 differentiation	50–60	PLPITPVXA...
D-Factor	Murine	Induction of M1 differentiation	40–67	SPLPITPVXA...
DIF-A & B	Human	Induction of M1 differentiation	51	PLPITPVXA...
DIA	Rat	Inhibition of ES cell differentiation	40–50	SPLPITPVXA
DRF	Murine	Inhibition of ES cell differentiation	40–50	nd
HSF-II & III	Human	Increased acute phase protein synthesis	32–39	nd
CNDF/CDF	Rat	Induction of cholinergic phenotype	45	SPLPITPVXA...
MLPLI	Human	Inhibition of lipoprotein lipase	50	SPLPITPVXA...
HILDA	Human	Stimulation of DA-1A proliferation	30–50	SPLPITPVXA...

[a]LIF, leukaemia inhibitory factor; D-factor, differentiation-inducing factor; DIF, differentiation-inducing factor; DIA, differentiation-inhibitory activity; DRF, differentiation-retarding factor; HSF, hepatocyte stimulating factor; CNDF/CDF, cholinergic neuronal differentiation factor; MLPLI, melanocyte derived lipoprotein lipase inhibitor; HILDA, human interleukin for DA-1A myeloid cells (see Ref. 1 and citations therein).

and adipocytes. For example, in the early 1970s neurons from neonatal rat dorsal root ganglia were shown to switch from an adrenergic to a cholinergic phenotype when exposed to medium conditioned by heart cells. Patterson's group recently isolated the protein responsible for this effect and showed it to be the rat homologue of LIF[24]. LIF is also able to promote the survival of embryonic neurons that differentiate from explanted neural crest and mature neurons isolated from embryonic dorsal root ganglia[25].

The acute phase response is an evolutionarily well conserved reaction to stress such as tissue damage. It involves the production in the liver of a defined set of proteins, including α_1-antitrypsin and fibrinogen. The nature of the hormones responsible for eliciting an acute phase response has been the focus of much research over the past 15 years. Interleukin 1 and tumour necrosis factor have been shown to stimulate the synthesis of a small subset of hepatic acute phase proteins[26]. Baumann and co-workers have demonstrated recently that three other polypeptide hormones, IL-6 (termed by them HSF-I), LIF (termed HSF-II and HSF-III) and IL-11 induce the synthesis of a much wider spectrum of acute phase proteins. The set of proteins stimulated by LIF, IL-6 and IL-11 is very similar, although in some cases the kinetics of stimulation appear to differ[27,28]. Interestingly, in mice that have elevated circulating levels of LIF, reduced serum albumin levels and an increased erythrocyte sedimentation rate are observed, both signs of an ongoing acute phase response[23].

A role for LIF in the regulation of lipid metabolism and the pathogenesis of tumour-related wasting disease (cachexia) has recently been suggested. SEKI is a cell line that was isolated from the melanoma of a patient with severe cachexia and causes a similar cachectic disease when transplanted into nude mice. Mori *et al.* demonstrated that SEKI cells secrete a protein (found by amino acid analysis to be LIF) that inhibited the action of lipoprotein lipase and thereby reduced the uptake of fatty acids by 3T3-L1 adipocytes[29]. That LIF was the molecule responsible for the tumour-related cachexia in mice, and possibly in the original patient, was further suggested by the dramatic and rapid loss of virtually all subcutaneous and abdominal fat in mice with experimentally elevated circulating LIF levels[22,23].

The meaning of LIF – the search for its physiological role

While interesting actions of LIF have been defined *in vitro* and a number of these have found pathological correlates *in vivo*, the normal *in vivo* function of LIF remains uncertain. Clearly, one of the major routes by which an understanding of the physiological role of LIF will be made is the analysis of the temporal and spatial pattern of expression of LIF and its receptor. A broad survey of adult mouse tissues by northern blot, RNase protection and polymerase chain reaction assays consistently failed to detect LIF mRNA. In addition, LIF is not normally detectable in the circulation of mice and, when injected intravenously, is cleared extremely rapidly ($t_{1/2}$ of 3–5 min; Ref. 30). These observations suggest that LIF expression is under tight control and that it may act primarily in a local environment rather than systemically. One such local site of LIF action has recently been defined.

When RNA was analysed from pregnant mice, LIF message was detected in the uterus at 4 days after mating, but neither earlier nor later (C. L. Stewart, pers. commun.). LIF expression was not dependent on the presence of embryos *per se*, since specific mRNA was also detected in the uterus 4 days after mating females with vasectomized males (C. L. Stewart, pers. commun.). Expression of LIF mRNA was localized, by *in situ* hybridization, to the metrial gland, a structure that secretes material into the uterine lumen and the presence of biologically active LIF in the fluid within the lumen suggests that LIF is indeed secreted (C. L. Stewart, pers. commun.). The situation is complicated further by the observation that the blastocyst stage embryos, present at 4 days of gestation, also contain LIF mRNA and secrete biologically active LIF[31]. Interestingly, during this critical period, the blastocyst moves from the oviduct into the uterus, hatches from the zona pellucida, implants in the uterine epithelium, begins to increase in overall size and the ICM differentiates into two of the primary germ layers, the ectoderm and the endoderm. It is clear that elucidation of the role of LIF at this stage of development requires a great deal of further work.

It seems logical that a molecule such as LIF, which acts on a broad spectrum of cells and which is harmful when present for extended periods in the circulation, should exert its effects in a local environment rather than systemically. The elucidation of other potential sites of LIF action will therefore require a careful temporal and spatial analysis of the pattern of LIF and LIF receptor expression. In addition, the effect of null mutations of the LIF gene and/or LIF receptor gene would complement studies on the effects of excess LIF and would help to define the physiological roles of this cytokine.

LIF free or bound – studies on the LIF receptor

As has been described, LIF is one of a growing group of hormones that are biologically promiscuous yet functionally redundant. The biochemical basis for pleiotropy and redundancy are not clear, however recent studies concerning the receptors for LIF and other cytokines appear to shed light on this problem.

LIF receptors, with similar characteristics are expressed on all biologically responsive cell types examined. These receptors are of high affinity (K_d = 20–80 pM) and are characterized by rapid association kinetics and, at 4°C, very

slow dissociation kinetics. LIF receptors expressed by responsive cells are also specific, since other cytokines, including IL-6 and G-CSF, that are structurally and functionally related to LIF, do not compete for binding (Refs 9, 20, 32; D. J. Hilton and N. A. Nicola, unpublished). Certain populations of activated macrophages express low affinity LIF receptors as well as high affinity receptors. In addition, the preparation of membranes from cells, such as ES cells, adipocytes and hepatocytes, that express only high affinity receptors results in the conversion of 40–60% of these to low affinity receptors. Moreover, upon solubilization of these membranes in detergent, receptors are recovered quantitatively, however all of these are of the low affinity type (see Fig. 2). Whether on the surface of cells, associated with membranes or in detergent solution, low affinity receptors differ from high affinity receptors only in the rate at which dissociation of LIF occurs (k_{off} = 0.3–0.8 min^{-1} versus 0.0004–0.0015 min^{-1}), the association rate of LIF with the two receptor types being indistinguishable (3–9 x 10^8 min^{-1} M^{-1}).

Recently, the receptors for human and murine LIF were cloned by expression[33]. The properties of the cloned receptor when expressed in COS cells paralleled that of the low affinity LIF receptor expressed by activated macrophages and present in detergent solution (Ref. 33; D. J. Hilton and N. A. Nicola, unpublished). Moreover the LIF receptor was found to be a member of the newly defined cytokine receptor family, which includes the receptors for growth hormone, prolactin, interleukins 2,3,4,5,6 and 7, CNTF, G-CSF, granulocyte–macrophage colony-stimulating factor (GM-CSF) and erythropoietin[34]. The features common to this family include the presence of one or two domains containing conserved cysteinyl residues and a seven-residue motif GXWSXWS. Structurally the cytokine receptors are distinct from G protein-linked receptors and receptors with tyrosine kinase activity, and thus the mechanism by which they transduce their signal is not known.

The structural similarity between the receptor for LIF and other members of the cytokine receptor family also extends to a functional similarity. Like LIF, high and low affinity receptors for GM-CSF, IL-3 and IL-5 are normally expressed by responsive cells, but the expression of the cloned receptors yields only low affinity receptors (reviewed in Ref. 35). In the case of GM-CSF the generation of low affinity receptors, at the expense of high affinity receptors, is also observed on preparation and solubilization of membranes[35]. The mechanism by which high affinity receptors for GM-CSF, IL-3, IL-5 and IL-6 are generated has recently been elucidated. A human molecule termed KH97, cloned on the basis of its cross-hybridization to the murine IL-3 receptor cDNA, failed to bind human IL-3 or any other cytokine examined, but was found to interact with the binding subunit of the human GM-CSF receptor to generate a complex that bound GM-CSF with a high affinity[35]. Subsequently it has been found that the same affinity 'converter' molecule interacts with the low affinity receptors for IL-3 and IL-5, to form a complex that binds these cytokines with a high affinity[35]. A similar mechanism occurs with the generation of high affinity IL-6 receptors through the interaction of the binding subunit with gp130[36]. In the case of IL-6, the gp130 molecule is essential for the transduction of a biological signal. Interestingly, the molecules that convert receptors to a high affinity form, are themselves members of the same cytokine receptor family[35,36].

A similar model for the LIF receptor would clearly explain a number of the observations described above (Fig. 2). For example, variation in the ratio of the LIF binding subunit to the putative converter molecule would explain the occurrence of cells expressing only high, high and low or only low affinity LIF receptors and the ability of the cloned receptor, when expressed alone, to yield low affinity but not high affinity receptors. In addition, if the interaction between the two components is less favoured when cell membrane integrity is disrupted, one might expect to observe a decrease in the ratio of high to low affinity receptors.

The structural relatedness of the binding subunits of cytokine receptors and the ability of these to interact with common affinity 'converter' molecules (which also appear to be central to signal transduction) may also explain why distinct cytokines elicit common actions on certain cell types. For example, the similar action of LIF, G-CSF and IL-6 on M1 cells might be explained if each receptor interacts with the gp130 component of the IL-6 receptor or a structurally related species to yield a common second messenger cascade and, ultimately, a common biological outcome (Fig. 3). It is possible that a single receptor can interact with several 'converter' molecules to generate different second messenger cascades and hence biological outcome (Fig. 3). In this way the different effects exerted by LIF on M1 monocytic leukaemia cells, ES cells and megakaryocytes could be ex-

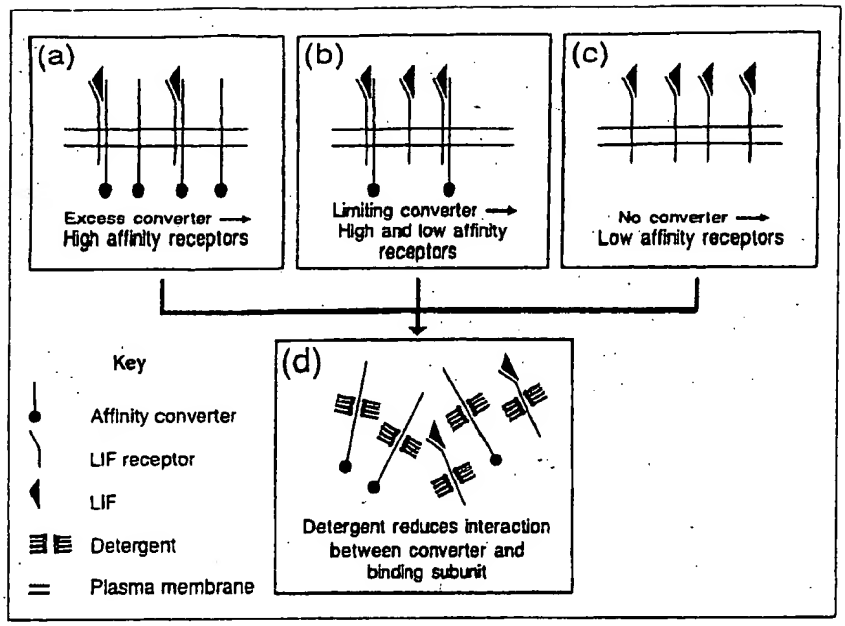

Figure 2
A model of LIF receptor structure and function: a scenario in which high affinity LIF receptors are generated via interaction of a low affinity binding subunit with a 'converter' molecule would explain many of the properties of LIF binding to its receptor in different environments. The model is discussed in detail in the text.

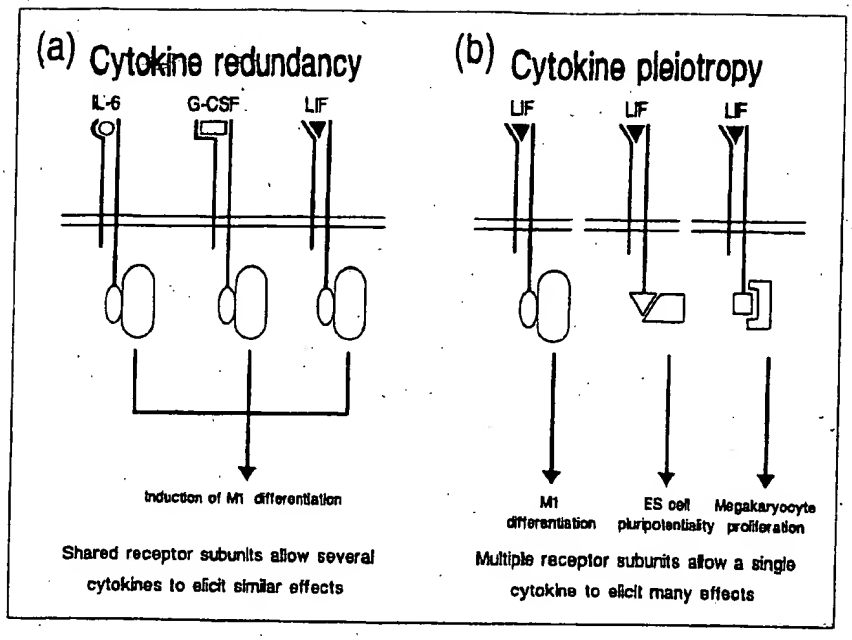

Figure 3

A possible structural basis for the pleiotropic and degenerate nature of cytokine action: polypeptide hormones, such as LIF, exert a broad range of biological effects, many of which are shared with other cytokines. The receptors for many cytokines share common structural motifs, and interact with common 'converter' molecules, that may be responsible for generating high affinity receptor and transducing a biological response[35,36]. Clearly, a model in which the binding subunits of the LIF, IL-6 and GM-CSF receptors interacted with a common second subunit would explain the similar ability of these cytokines to induce M1 differentiation (a). Moreover, if the receptor for LIF could interact with several different second subunits, the pleiotropic nature of its biological effects might be explained (b).

plained. It should be stressed that this explanation for the basis of pleiotropic cytokines, although attractive, is purely speculative and alternatively the divergence of intracellular signalling pathways leading to different biological effects may occur downstream of the receptor, in the cytoplasm or nucleus.

Conclusions and future perspectives

LIF has many interesting, and seemingly contradictory, functions both *in vitro* and *in vivo*. From a technological viewpoint, LIF has proven useful because of its ability to maintain the pluripotentiality of ES cells, and may one day prove useful in the clinical setting because of its ability to increase the circulating numbers of platelets. The physiological role of LIF still, however, remains unclear. Progress towards understanding this role awaits a thorough analysis of the temporal and spatial pattern of expression of LIF and its receptor, as well as an analysis of the effect of null mutations of the genes of LIF and its receptor.

The cloning of the LIF receptor and demonstration of its relatedness to other cytokine receptors suggests certain models of receptor structure that are consistent with the equilibrium and kinetic properties of LIF binding to a wide range of cell types. In addition, it is possible that the shared features of various cytokine receptors provide the structural basis for the overlapping action of many cytokines like LIF, IL-6, GM-CSF and oncostatin-M. The route by which these receptors transduce their signal remains unknown, as does the point at which signal transduction pathways diverge to yield apparently opposite actions of a single hormone on different cell types. The cloning of binding subunits and affinity 'converter' subunits of several cytokine receptors makes the understanding of these problems closer at hand.

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